

Induction of interleukin-8 gene expression by black-pigmented *Bacteroides* in human pulp fibroblasts and osteoblasts

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Abstract

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Aim To investigate the effect of black-pigmented *Bacteroides* on the expression of interleukin (IL)-8 gene in human pulp fibroblasts and osteoblasts.

Methodology The supernatants of *Porphyromonas endodontalis*, *P. gingivalis* and *Prevotella intermedia* were used to evaluate IL-8 gene expression in human pulp fibroblasts and osteoblasts. The levels of mRNAs were measured by the quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

Results Investigations of the time-dependence of IL-8 mRNA expression in black-pigmented *Bacteroides*-treated

pulp fibroblasts and osteoblasts revealed a rapid accumulation of the transcript after 2 h of exposure, and remained elevated throughout the 24-h incubation period. In addition, IL-8 mRNA gene expression was also found in human osteoblasts stimulated with black-pigmented *Bacteroides*. However, black-pigmented *Bacteroides* was found to be more effective in the induction of IL-8 mRNA gene expression in osteoblasts than in pulp fibroblasts ($P < 0.05$).

Conclusions Black-pigmented *Bacteroides* are capable of amplifying the local immune response and promoting pulpal/periapical tissue inflammation by stimulating pulp fibroblasts and osteoblasts to express IL-8.

Keywords: black-pigmented *Bacteroides*, IL-8, osteoblasts, pulp fibroblasts.

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Introduction

The major causes of pulpal and periapical lesions are microbial infection of the dental pulp and the persistence of bacterial infection in the root-canal system. Black-pigmented *Bacteroides* have been identified with increasing frequency from odontogenic abscesses (Jim *et al.* 1989). *Porphyromonas endodontalis*, *P. gingivalis* and *Prevotella intermedia* have been documented in human dental pulpal and periapical diseases (Van Winkelhoff *et al.* 1985, Sundqvist *et al.* 1989). These bacteria have been demonstrated to express a variety of factors that play many roles

in pathogenicity, including fimbriae, proteases and lipopolysaccharide endotoxins. The invasion of host tissue by microbes or their by-products frequently induce a variety of immunopathological reactions.

Various cytokines play important roles and regulate the intensity and duration of the immune response against potentially pathogenic agents. Chemokines, a family of chemotactic cytokines, are low-molecular-weight proteins, which can stimulate recruitment of leucocytes. Members of the chemokine family have specificity to recruit well-defined subsets of leucocytes. Thus, chemokine expression can account for the presence of different cell types observed in various disease states. Interleukin (IL)-8 is a chemokine produced by a variety of tissue and blood cells, and is a potent inducer of neutrophil chemotaxis and activation (Van Damme 1991, Baggiolini *et al.* 1994). Aberrant and persistent production of IL-8 has been demonstrated in various inflam-

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matory diseases, including pulpal inflammation (Huang *et al.* 1999) and periapical lesions (Shimauchi *et al.* 2001). However, the precise mechanisms of pulpal and periapical inflammations have not been well elucidated.

Pulp fibroblasts and osteoblasts are considered as cells primarily concerned with providing physical barriers and structural components in pulpal and periapical tissues. These cells may be important in the recruitment of immune cells, and contribute to the inflammation. To date, the interactions of bacteria recovered from root canals and human pulp fibroblasts as well as osteoblasts are still not fully understood. The aim of this study was to investigate the IL-8 expression by human pulp fibroblasts and osteoblasts stimulated with black-pigmented *Bacteroides* (*P. endodontalis*, *P. gingivalis* and *P. intermedia*) by reverse transcriptase-polymerase chain reaction (RT-PCR) assay.

Materials and methods

Cell culture

Human pulp fibroblasts were cultured using an explant technique as described previously by Chang *et al.* (2000) and Chang & Chou (2001). Briefly, impacted third molars were obtained from healthy patients of the Oral Medicine Center, Chung Shan Medical University Hospital, Taichung, Taiwan. Teeth were sectioned horizontally below the cemento-enamel junction with a number 330 high-speed bur with water spray. The pulp tissue was removed aseptically in a laminar flow environment, rinsed with Hanks' buffered saline solution and placed in a 60-mm dish. Pulp tissue was minced with a blade into small fragments and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% foetal calf serum (FCS; Gibco Laboratories) and antibiotics (100 U mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin and 0.25 µg mL⁻¹ of fungizone). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5 min, and aliquots of separated cells were subcultured. Cell cultures between the third and eighth passages were used in this study.

U2-OS (American Tissue Type Collection HTB 96), derived from human osteogenic sarcoma, was cultured in DMEM supplemented with 10% FCS, 100 µg mL⁻¹ of streptomycin, 100 mg mL⁻¹ of penicillin at 37 °C in humidified incubator under ambient pressure air atmosphere containing 5% CO₂ (Huang *et al.* 2003). Confluent cells were detached with 0.25% trypsin and 0.05% EDTA

for 5 min, and aliquots of separated cells were subcultured. The cells were subcultured at 1 : 4 splits every third day.

Bacterial strains and preparation of supernatants

The bacterial strains tested were *P. endodontalis* (ATCC 27067), *P. gingivalis* (ATCC 33277) and *P. intermedia* (ATCC 25611). They were maintained in brain–heart infusion broth prerduced anaerobically, sterilized and supplemented with 5 mg L⁻¹ haemin and 0.5 mg L⁻¹ menadione. The density of the inoculum prepared in brain–heart infusion broth was adjusted to a turbidity of 2 McFarland standard (6×10^8 colony-forming units mL⁻¹). After centrifugation, supernatants were filter-sterilized using a 0.2-µm filter and stored at –80 °C until used. The supernatants of *P. endodontalis*, *P. gingivalis* and *P. intermedia* were directly diluted in culture medium, and the final dilution was 1 : 100.

Treatments

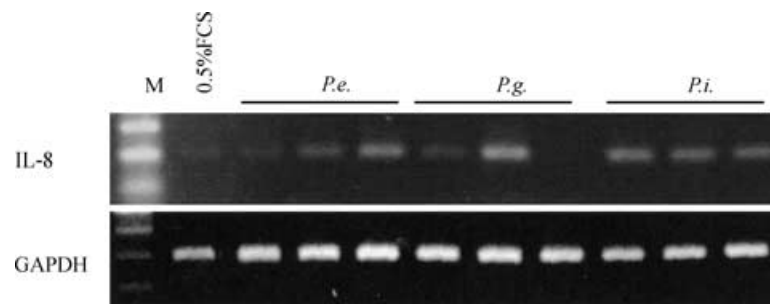
Confluent cells were trypsinized, counted and plated at a concentration of 1×10^5 cells in a 60-mm dish and allowed to achieve confluence. Cells arrested in G₀ by serum deprivation (0.5% FCS for 48 h) were generally used in these experiments (Chang *et al.* 2003a,b). Before treatment, the cells were washed with serum-free DMEM and immediately exposed for the indicated incubation times (2, 6 and 24 h) to the supernatants of *P. endodontalis*, *P. gingivalis* and *P. intermedia*, respectively. Cultures with 0.5% FCS were used as positive control.

Total RNA preparation and RT-PCR

Total RNA was prepared using TRIzol reagent (Gibco Laboratories) following the manufacturer's instructions. Single-stranded DNA was synthesized from RNA in a 15-µL reaction mixture containing 100 mg random hexamer and 200 units of Moloney murine leukaemia virus reverse transcriptase (Gibco Laboratories). The reaction mixture was diluted with 20 µL of water, and 3 µL of the diluted reaction mixture was used for the PCR. PCR reaction mixture contained 10 pmol of forward and reverse primers and 2 units of Tag DNA polymerase. Amplification was performed at 25 cycles for glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and 30 cycles for IL-8 in a thermal cycle. Each cycle consisted of 1 min of denaturation at 94 °C, 1 min of annealing at 57 °C and 1 min of extension at 72 °C. The sequences of primers employed are listed in Table 1

Table 1 Nucleotide sequence and size of the expected PCR products for oligonucleotide primers used for RT-PCR

Gene	Sequence	PCR product (bp)
GAPDH	Forward (5'-TCCTCTGACTTCAACAGCGACACC-3'); reverse (5'-TCTCTCTTCCTCTTGCTCTTGG-3')	207
IL-8	Forward (5'-CGATGTCAGTGCATAAAGACA-3'); reverse (5'-TGAATTCTCAGCCCTCTCAAAAA-3')	225

**Figure 1** Expression of IL-8 mRNA gene stimulated with black-pigmented *Bacteroides* (*P. endodontalis* (*P.e.*), *P. gingivalis* (*P.g.*) and *P. intermedia* (*P.i.*)) in human pulp fibroblasts by RT-PCR assays. M = DNA molecular size marker. Lane 1 represents control (0.5% FCS); lanes 2–4 represent *P.e.*: 2, 6 and 24 h, respectively; lanes 5–7 represent *P.g.*: 2, 6 and 24 h, respectively; lanes 8–10 represent *P.i.*: 2, 6 and 24 h, respectively.

(Hirose *et al.* 2001). The PCR products were analysed by agarose gel electrophoresis.

When the cells were probed for IL-8 mRNA production by RT-PCR, a 225-bp band for IL-8 was noted. These bands were consistent with the size as designed by the primer. When the band densities were measured and compared with the density of the band obtained for the housekeeping gene GAPDH, relative proportions of mRNA synthesis could be determined within each experiment. The intensity of each band after normalization with GAPDH mRNA was quantified by the photographed gels with a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA, USA). Each densitometric value was expressed as the mean $\pm$ SD.

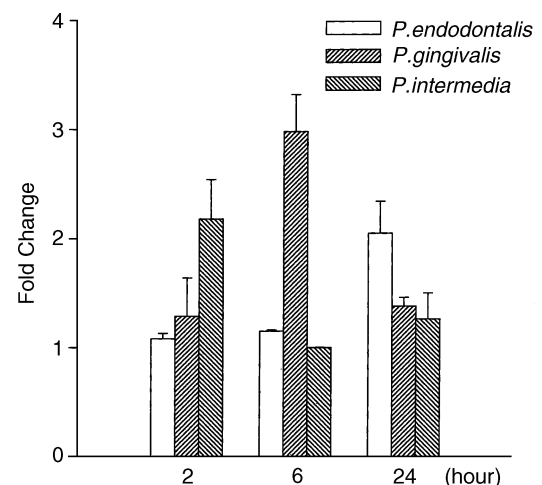
Statistical analysis

All assays were repeated thrice to ensure reproducibility. Statistical analysis was carried out by one-way ANOVA. Tests of differences of the treatments were analysed by Duncan's test, and a value of $P < 0.05$ was considered statistically significant.

Results

Black-pigmented *Bacteroides* were found to induce IL-8 gene expression in human pulp fibroblasts (Fig. 1). However, human pulp fibroblasts resting in 0.5% FCS did not express any detectable level of IL-8 gene.

Densitometric analysis of the IL-8 mRNA gene expression after normalization by GAPDH demonstrated that IL-8 mRNAs increased about 1.1-, 1.3- and 2.2-fold after exposure to *P. endodontalis* for 2, 6 and 24 h, respectively (Fig. 2). The levels of the IL-8 mRNAs increased about 1.2-, 3.0- and 1.0-fold after exposure to *P. gingivalis* for 2, 6 and 24 h, respectively (Fig. 2). The levels of the IL-8

**Figure 2** Levels of IL-8 mRNA gene treated with black-pigmented *Bacteroides* were measured by AlphaImager 2000 (Alpha Innotech). Optical density values expressed as the mean $\pm$ SD was obtained from three independent experiments.

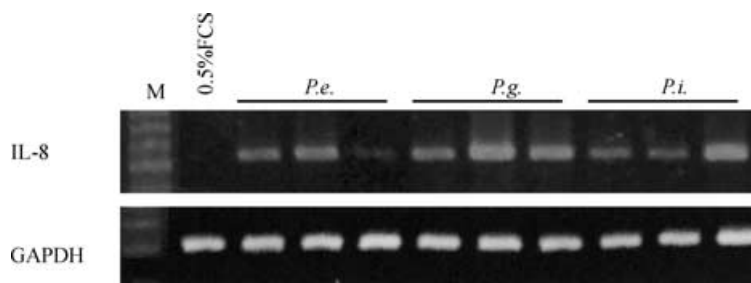


Figure 3 Expression of IL-8 mRNA gene stimulated with black-pigmented *Bacteroides* (*P.e.*, *P.g.* and *P.i.*) in human osteoblasts by RT-PCR assays. M = DNA molecular size marker. Lane 1 represents control (0.5% FCS); lanes 2–4 represent *P.e.*: 2, 6 and 24 h, respectively; lanes 5–7 represent *P.g.*: 2, 6 and 24 h, respectively; lanes 8–10 represent *P.i.*: 2, 6 and 24 h, respectively.

mRNAs increased about 2.1-, 1.4- and 1.3-fold after exposure to *P. intermedia* for 2, 6 and 24 h, respectively (Fig. 2).

Similar induction patterns were also found in human osteoblasts stimulated with black-pigmented *Bacteroides* (Fig. 3). However, significantly higher IL-8 mRNA levels were found in osteoblasts than in pulp fibroblasts ($P < 0.05$). As shown in Fig. 4, the levels of the IL-8 mRNAs increased about 2.6-, 4.0- and 1.3-fold after exposure to *P. endodontalis* for 2, 6 and 24 h, respectively. The levels of the IL-8 mRNAs increased about 2.8-, 4.6- and 2.7-fold after exposure to *P. gingivalis* for 2, 6 and 24 h, respectively. The levels of the IL-8 mRNAs increased about 1.9-, 1.6- and 2.9-fold after exposure to *P. intermedia* for 2, 6 and 24 h, respectively.

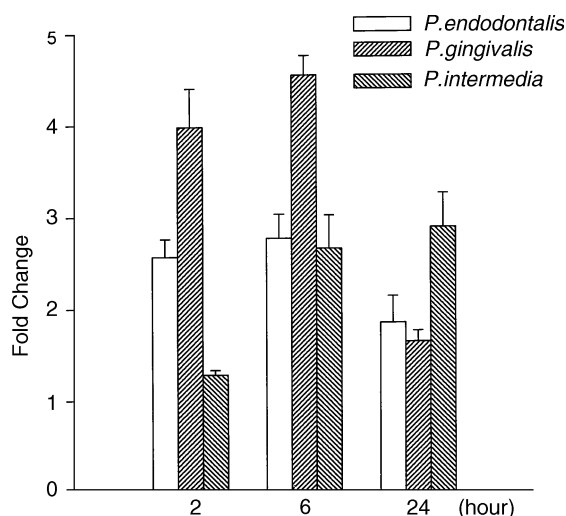


Figure 4 Levels of IL-8 mRNA gene treated with black-pigmented *Bacteroides* were measured by AlphaImager 2000 (Alpha Innotech). Optical density values expressed as the mean $\pm$ SD was obtained from three independent experiments.

Discussion

Cytokines and inflammatory mediators share a multitude of biological activities that contribute to a coordinated response of immune system to injury or bacterial challenge. Challenge of human pulp fibroblasts with black-pigmented *Bacteroides* has been documented to result in mRNA expression and/or secretion of immunoregulatory molecules such as matrix metalloproteinases (Chang *et al.* 2002), cyclooxygenase-2 enzyme (Chang *et al.* 2003c), plasminogen activator (Yang *et al.* 2003a) and IL-6 (Yang *et al.* 2003b). Dysregulated production of these inflammatory mediators may lead to an excessive local amplification of the immune response, which may be partly responsible for pulpal and periapical tissue destruction.

IL-8 has been demonstrated in inflamed pulp tissue (Huang *et al.* 1999) as well as periapical lesions (Shimauchi *et al.* 2001). This chemokine may, in turn, initiate and augment inflammatory processes in pulpal and periapical lesions. The experiments reported here demonstrated that black-pigmented *Bacteroides*-induced human osteoblasts express IL-8 mRNA gene. This appears to be the first study to report such an effect of black-pigmented *Bacteroides* on IL-8 gene expression in human osteoblasts. In addition, this similar pattern was found in human pulpal fibroblasts. The results are in agreement with previous studies that endodontic pathogens can stimulate IL-8 production in human pulp fibroblasts (Nagaoka *et al.* 1996) and mononuclear cells (Jiang *et al.* 1998). IL-8 may exemplify the ability of cells to respond in a diverse fashion and influence the outcome of the inflammatory response in tissues. Stimulation of IL-8 production may be one of the pathogenetic mechanisms of pulpal and periapical inflammation challenged with black-pigmented *Bacteroides* in resident cells.

Amongst the three supernatants of bacterial cultures tested, the amounts of IL-8 mRNA gene expression were different between human pulp fibroblasts and osteoblasts. The induction patterns were dependent on the bacteria and the cells tested. The reason for different activation is still not clear in this study. One possible way whereby the supernatants from various bacteria have been shown to differ is in the structure of their lipid A as well as their polysaccharide chain (Novotny 1984). Second, this activation may result from the different origins of the cells, and the cellular effect of stimulation may not necessarily be comparable in all tissues.

LPS from Gram-negative bacteria are powerful immuno-stimulatory agents which initiate the local synthesis and release of proinflammatory cytokines from the cells of immune system (Dijkmans *et al.* 1990). Macrophages were originally identified as being the predominant cellular sources of these cytokines (Dinarello 1989). In this study, human pulp fibroblasts and osteoblasts were found to express IL-8 when stimulated by black-pigmented *Bacteroides*. Thus, the roles of pulp fibroblasts and osteoblasts not only provide structural support, but also may function as accessory immune cells and play an important role in the initial inflammatory reaction as well as in the amplification of immune response.

Our results have demonstrated that human pulp fibroblasts and osteoblasts may contribute significant levels of IL-8 in the pulpal and periapical milieu in response to bacterial challenge. Taken together, besides the role of macrophages and other immune cells, the role of pulp fibroblasts and osteoblasts in the regulation of IL-8 must be taken into account when considering the clinical manifestations of pulpal and periapical diseases. Further understanding the role of pulp fibroblasts and osteoblasts in IL-8 expression would enable us to better explain the pathophysiology of these diseases.

Conclusions

Bacterial infection of the pulp and root-canal system leads to the recruitment of immuno-competent cells in the apex, and stimulates inflammatory cell responses to produce a variety of cytokines. The present study demonstrated that IL-8 gene expression by human pulp fibroblasts and osteoblasts was induced by black-pigmented *Bacteroides* (*P. endodontalis*, *P. gingivalis* and *P. intermedia*). These results suggest that pulp fibroblasts and osteoblasts may act as immuno-responsive cells and can elaborate IL-8 upon stimulation with black-pigmented *Bacteroides*.

Acknowledgements

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